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Lipid-functionalized Dextran Nanosystems to Overcome Multidrug Resistance in Cancer

A Pilot Study

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Abstract

Background The toxicity of anticancer agents and the difficulty in delivering drugs selectively to tumor cells pose a challenge in overcoming multidrug resistance (MDR). Recently, nanotechnology has emerged as a powerful tool in addressing some of the barriers to drug delivery, including MDR in cancer, by utilizing alternate routes of cellular entry and targeted delivery of drugs and genes. However, it is unclear whether doxorubicin (Dox) can be delivered by nanotechnologic approaches.

Questions/Purposes We asked whether (1) Dox-loaded lipid-functionalized dextran-based biocompatible nanoparticles

(Dox/NP) can reverse MDR, (2) Dox/NP has more potent cytotoxic effect on MDR tumors than poly(ethylene glycol)-modified liposomal Dox (PLD), and (3) multidrug resistance protein 1 (*MDR1*) small interfering RNA loaded in these nanoparticles (siMDR1/NP) can modulate MDR.

Methods To create stable Dox/NP and siMDR1/NP, we used two different lipid-modified dextran derivatives. The effect of Dox or Dox/NP was tested on drug-sensitive osteosarcoma (KHOS) and ovarian cancer (SKOV-3) cell cultures in triplicate and their respective MDR counterparts KHOS_{R2} and SKOV-3_{TR} in triplicate. We determined the effects on drug retention, transfection efficacy of siMDR1/NP, and P-glycoprotein expression and the antiproliferative effect between Dox/NP and PLD in MDR tumor cells.

Results Fluorescence microscopy revealed efficient uptake of the Dox/NP and fluorescently tagged siMDR1/NP. Dox/NP showed five- to 10-fold higher antiproliferative activity at the 50% inhibitory concentration than free Dox in tumor cells. Dox/NP showed twofold higher activity than PLD in MDR tumor cells. siMDR1/NP (100 nM) suppressed P-glycoprotein expression in KHOS_{R2}.

Conclusions Dextran-lipid nanoparticles are a promising platform for delivering Dox and siRNAs.

Clinical Relevance Biocompatible dextran-based nanoparticles that are directly translatable to clinical medicine

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may lead to new potential therapeutics for reversing MDR in patients with cancer.

Introduction

The treatment of many cancers using chemotherapy still remains a great challenge. Although the cure rate of patients with diseases such as localized osteosarcoma (OS) and ovarian cancers has improved due to the introduction of combinatorial chemotherapy [6, 34], a large proportion of patients still respond poorly to drug treatment and have a high risk of local tumor relapse and metastasis to distant sites, even after multimodality treatments [2, 14, 56].

One of the major causes of the hampered efficacy of chemotherapy is the eventual development of multidrug resistance (MDR). MDR is considered one of the most important hindrances of curative therapies in humans [41]. Cancer cells almost invariably emerge to become resistant to a variety of anticancer drugs by employing a host of different mechanisms [15]. MDR in cancer is frequently associated with the overexpression of active transporters that act as drug efflux pumps. It has been well documented that P-glycoprotein (P-gp), the gene product of the multi-drug resistance protein 1 gene (*MDR1*, *ABCB1*), has been a major component of the drug efflux pump that prevents intracellular accumulation of anthracycline drugs [13]. Although reversal of MDR has been reported with several agents, unfortunately, they were relatively nonspecific and had low potency and/or adverse toxicity [4, 21, 24, 43, 45]. Consequently, the development of alternative therapeutic strategies employing more efficient delivery systems is imperative to overcome MDR.

On the other hand, silencing of *MDR1* mRNA expression using small interfering RNA (siRNA) for *MDR1* (siMDR1) is one of the most powerful approaches for reversing MDR [32]. However, RNA interference mechanisms also confront several challenges that hinder their clinical translation. For instance, the cellular uptake of siRNA is limited due to its poor membrane permeability, and in general, siRNAs are also prone to rapid degradation by nucleases [35]. Although commercially available transfection agents effectively protect and deliver siRNA in vitro, they have limited clinical utility because of their inherent toxicities. Thus, a more safe and effective delivery system for clinical use of siRNAs is warranted.

Recently, nanotechnology has gained tremendous interest for overcoming the limitations of conventional drug and gene delivery problems [9, 54]. Among the several reported systems, polymer-conjugated drugs and polymeric nanoparticle-based advanced delivery systems are gaining more attention because of their superior and distinct advantages, including the ability to overcome MDR in cancers [31].

Furthermore, polymeric nanoparticles can not only be easily tailored to stably encapsulate drug payloads or conveniently self-assembled into nanoparticles but can also be designed with spatial and temporal control based on the nature and choice of the biodegradable polymer used for constructing self-disintegrating nanosystems [5, 10, 52, 55]. Among the commercially available natural polymers, dextran and its derivatives have been widely studied and used as polymeric drug carriers due to their biocompatibility and biodegradability [49].

We therefore explored the role of lipid-functionalized dextran-based nanosystems to overcome MDR. We asked whether (1) doxorubicin (Dox)-loaded lipid-functionalized dextran-based biocompatible nanoparticles (Dox/NP) can reverse MDR, (2) Dox/NP shows more potent cytotoxic effect on MDR tumors than poly(ethylene glycol) (PEG)-modified liposomal Dox (PLD), and (3) *MDR1* siRNA-loaded nanoparticles (siMDR1/NP) can modulate MDR.

Materials and Methods

To explore whether nanoparticles can overcome MDR in cancer, we first tested whether our formulation of Dox and siMDR1 using a lipid-modified dextran derivative resulted in formation of stable nanoparticles (Dox/NP and siMDR1/NP, respectively) (Fig. 1). We analyzed subcellular distribution of Dox in OS (KHOS, KHOS_{R2}) and ovarian cancer cells (SKOV-3, SKOV-3_{TR}) treated with Dox alone or Dox/NP using fluorescent microscopy and Dox and Dox/NP cytotoxicity by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays. Then, we compared the antiproliferative effect of Dox/NP and PLD in MDR tumor cells. Finally, we assessed the transfection efficacy of AF488-tagged siMDR1/NP on KHOS_{R2} cells and evaluated the effect of siMDR1/NP on P-gp expression. Each cell condition was cultured in triplicate and each experiment was repeated at least three times.

We obtained dextran (MW ~ 40 kDa) and other reagents from Sigma-Aldrich Chemical Co (St Louis, MO, USA) and Acros Organics (Parsippany, NJ, USA) and used them as obtained. We synthesized the clickable dextran, thiol-modified dextran, and the PEG-modified dextran according to a previously reported procedure [1]. The dextran acrylate and lipid-modified dextran were formulated based on the published procedure of Susa et al. [48].

The Dox/NP were formulated based on the procedure of Abeylath and Amiji [1]. Briefly, the drug-loaded nanoparticles were prepared by self-assembly of dextran blocks. Dextran-C6 lipid (19.5 mg), dextran-thiol (15 mg), and dextran-PEG (15 mg) were synthesized separately and then dissolved together in 4 mL water; Dox (0.5 mg) in water (1 mL) was added to it and vortexed for 5 minutes. The

Fig. 1 A flow diagram shows the experiments we performed using Dox/NP and siMDR1/NP.

Formulation of lipid-modified dextran nanoparticles as carriers for Dox and siMDR1

Cell lines: Drug-sensitive cells and their MDR counterparts

Osteosarcoma: KHOS and KHOS_{R2} Ovarian Cancer: SKOV-3 and SKOV-3_{TR}

(1) Can Dox/NP reverse MDR?

To assess cellular Dox uptake

KHOS, KHOS_{R2}, SKOV-3, and SKOV-3_{TR}

(1 × 10⁵ cells/well in 12-well plates, n = 3 wells/cell line)

Fluorescence microscopic examination after 3 hours' incubation with Dox or Dox/NP

In vitro cytotoxicity assay (MTT assay)

KHOS, KHOS_{R2}, SKOV-3, and SKOV-3_{TR}

(1500 cells/well in a 96-well plate, n = 3 wells/concentration)

5 days' incubation with Dox or Dox/NP

(0.001, 0.01, 0.03, 0.06, 0.1, 0.3, 0.6, 1, 3, 6, 10 μM)

(2) Does Dox/NP have more potent cytotoxic effect on MDR tumors than PLD?

In vitro cytotoxicity assay (MTT assay)

KHOS_{R2} and SKOV-3_{TR}

(1500 cells/well in a 96-well plate, n = 3 wells/concentration)

5 days' incubation with Dox/NP or PLD

(0.001, 0.01, 0.03, 0.06, 0.1, 0.3, 0.6, 1, 3, 6, 10 μM)

(3) Can siMDR1/NP modulate MDR?

To assess cellular siMDR1 uptake

KHOS_{R2} (1 × 10⁵ cells/well in 12-well plates, n = 3 wells/condition)

Fluorescence microscopic examination after 2, 4, and 24 hours' incubation with 100 nM AF488-tagged siMDR1/NP, siMDR1 + RNAiMAX, or siMDR1 only

To assess P-gp expression

KHOS_{R2} (1 × 10⁵ cells/well in 12-well plates; n = 2 wells/condition)

Western blotting after 48 hours' incubation with 100 nM siMDR1/NP, siMDR1 + RNAiMAX, negative control siRNA + RNAiMAX, nanoparticles only, or cells only

mixture was lyophilized and the fluffy powder was dispersed in 5 mL deionized water. The particle size and zeta potentials of the self-assembled nanoparticles were measured using a dynamic light scattering (DLS) instrument as described below.

The siMDR1/NP were formulated based on the procedure of Susa et al. [48]. Briefly, stock solutions of 5 mg/mL PEG-thiol, octylamine-modified dextran, and thiol-dextran were prepared in deionized water. A stock solution of 2 mM siRNA solution was prepared using RNase-free water. A 12-mL solution of the stock siMDR1 was taken in a tube and 40 μL thiol-dextran was first added and mixed well using a vortex shaker. It was then incubated for 5 minutes. To this mixture, 40 μL dextran-octylamine was then added and incubated for another 5 minutes. Finally,

40 μL PEG-thiol was added and incubated for another 15 minutes to form the hydrophilic shell of the nanoparticles. The nanoparticles were lyophilized to yield a fluffy powder. This method of sequential addition and lyophilization was used for the stable entrapment of the siRNA in the dextran hydrogel network. siRNAs at concentrations of 10, 30, 50, and 100 nM were prepared by adjusting the stock solution of siRNA while preparing the dextran nanoparticles. The particle size and zeta potentials of the nanosystems so formed were measured using a DLS instrument as described below.

We characterized the Dox/NP and siMDR1/NP for size and size distribution using a electrophoretic DLS instrument (Zetasizer[®] 3000HS; Malvern Instruments Ltd, Malvern, UK) at a 90° fixed angle and at 25°C. For zeta

potentials, default parameters of dielectric constant, refractive index, and viscosity of water were used based on the electrophoretic mobility of the nanoparticles. For the zeta potential measurements, a specially designed electrophoretic cell was used and the average surface charge was measured.

We obtained the human OS cell lines KHOS and the MDR (P-gp)-expressing cell line KHOS_{R2} from the National Hellenic Research Foundation (Athens, Greece) and the human ovarian cancer cell line SKOV-3 from the American Type Culture Collection (Manassas, VA, USA). The MDR SKOV-3_{TR} was established as reported previously [25]. All cell lines were cultured in RPMI 1640 medium with 10% fetal bovine serum and 100 U/mL penicillin incubated at 37°C in 5% CO₂-95% air atmosphere. MDR cell lines were continuously cultured in 0.1 µM Dox (KHOS_{R2}) or 0.1 µM taxol (SKOV-3_{TR}).

We obtained Dox and PLD (Doxil®; Janssen Biotech Inc, Horsham, PA, USA) from Massachusetts General Hospital as unused residual clinical materials. The anti-P-gp1 monoclonal antibody C219 was purchased from Signet (Dedham, MA, USA). The human β-actin monoclonal antibody and MTT reagents were purchased from Sigma-Aldrich. The siRNA sequence targeting *ABCB1* (Genebank Accession Number NM_00927) corresponded to coding regions of these genes. siMDR1 (sense strand, 5'-GACCAU AAAUGUAAGGUUU-3' and 5'-AAACCUUACA UUGGUC-3') was purchased from Sigma-Aldrich. Negative control siRNA (sense strand, 5'-GGGUAUCGACGAUUAC AAAUU-3') was obtained from Qiagen (Valencia, CA, USA). Fluorescently tagged siRNA (sense strand, 5'-GCUU AACACCCGACUUAUUAUUTT-3') for *MDR1* with a 3' AF488 modification was synthesized and provided by Qiagen. Although sequences were different between tagged and untagged siMDR1 purchased from different companies, they functioned well.

For cellular Dox uptake studies, we seeded KHOS, KHOS_{R2}, SKOV-3, and SKOV-3_{TR} cells ($n = 3$ wells/cell line; 1×10^5 cells/well) in 12-well plates and incubated them for 24 hours. After the incubation, either Dox alone or Dox/NP was added and the cells were incubated for another 3 hours. To stain nuclei, 1 µg/mL Hoechst 33342 (Life Technologies Corp, Grand Island, NY, USA) was used. After incubation, the cells were visualized using a Nikon Eclipse Ti-U fluorescence microscope (Nikon Instruments, Inc, Melville, NY, USA) equipped with a SPOT RT™ digital camera (Diagnostic Instrument, Inc, Sterling Heights, MI, USA).

To assess the cellular uptake of siMDR1, we seeded KHOS_{R2} cells ($n = 3$ wells/condition; 1×10^5 cells/well) in 12-well plates and incubated them with 100 nM AF488-tagged siMDR1/NP over 24 hours. AF488-tagged siMDR1 plus Lipofectamine™ RNAiMAX Transfection Reagent

(Life Technologies Corp) and AF488-tagged siMDR1 alone were used as positive and negative controls, respectively. After 2-, 4-, and 24-hour incubation, images were acquired by fluorescence microscopy.

We assayed in vitro cytotoxicity using the MTT assay. Briefly, we seeded KHOS, KHOS_{R2}, SKOV-3, and SKOV-3_{TR} cells ($n = 3$ wells/concentration; 1500 cells/well) (Dox versus Dox/NP) or KHOS_{R2} and SKOV-3_{TR} cells ($n = 3$ wells/concentration; 1500 cells/well) (Dox/NP versus PLD) in 96-well plates. Cells were cultured in medium with 0.001, 0.01, 0.03, 0.06, 0.1, 0.3, 0.6, 1, 3, 6, and 10 µM concentrations of Dox alone, Dox/NP, or PLD. After culturing for 5 days, 10 µL MTT (5 mg/mL in phosphate-buffered saline) was added to each well and the cells were incubated for 4 hours. After dissolving the resulting formazan product with acid isopropanol, the absorbance was analyzed on a SpectraMax® Microplate Spectrophotometer (Molecular Devices LLC, Sunnyvale, CA, USA) at a wavelength of 490 nm [47, 49, 59].

To evaluate the effect of siMDR1/NP on P-gp expression, we seeded KHOS_{R2} ($n = 2$ wells/condition; 1×10^5 cells/well) in 12-well plates and incubated them with 100 nM AF488-tagged siMDR1/NP over 48 hours. AF488-tagged siMDR1 plus Lipofectamine™ RNAiMAX Transfection Reagent, siRNA plus Lipofectamine™ RNAiMAX Transfection Reagent, nanoparticles only, and cells only were used as controls. P-gp was analyzed in total cell lysates. Protein lysates from cells were generated through lysis with $1 \times$ radioimmunoprecipitation assay lysis buffer (Upstate Biotechnology, Charlottesville, VA, USA). The protein concentration was determined by a bovine serum albumin assay (Bio-Rad Laboratories Inc, Hercules, CA, USA), using a Beckman DU-640 spectrophotometer (Beckman Instruments Inc, Columbia, MD, USA). For Western blotting, 25 µg of total protein was processed on NuPAGE® 4–12% Bis-Tris Gel (Life Technologies Corp) and transferred to pure nitrocellulose membrane (Bio-Rad). After an overnight incubation with primary antibodies in Tris-buffered saline (pH 7.4, with 0.1% Tween® 20), the signal was generated through incubation with relevant secondary antibodies (Bio-Rad) incubated in Tris-buffered saline (pH 7.4, with 5% nonfat milk and 0.1% Tween® 20) at 1:2000 dilution for 1 hour at room temperature. Blots were detected using SuperSignal® West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc, Waltham, MA, USA).

We report continuous data as a mean $\pm$ SD. We checked the normality of the data using the Kolmogorov-Smirnov test and found the null hypothesis of homoscedasticity was rejected in some data. We therefore determined differences in the antiproliferative effect of each cell line (KHOS, KHOS_{R2}, SKOV-3, SKOV-3_{TR}) cultured with Dox alone and Dox/NP using a Welch t-test. Differences in the antiproliferative effect in MDR tumor

cells (KHOS_{R2}, SKOV-3_{TR}) treated with Dox/NP and PLD were also assessed by Welch t-test.

Results

The formulation of Dox and siMDR1 using a lipid-dextran derivative resulted in formation of stable nanoparticles after lyophilization. The resuspended nanoparticles in deionized water revealed mean particle size and zeta potentials of 171 ± 2 nm and -1.21 ± 1.31 mV, respectively, for Dox/NP and 101 ± 3 nm and -0.22 ± 2.21 mV, respectively, for siMDR1/NP. Dox by itself possessed an intrinsic red fluorescence, and cell nuclei were stained with a nuclear-specific dye emitting blue fluorescence. When MDR cells were treated with Dox alone, the drug was primarily concentrated in the cytoplasm, and a very low level of fluorescence was observed in the nucleus (Fig. 2). On the other hand, MDR cells treated with Dox/NP showed increased fluorescence in the nucleus and cytoplasm. The subcellular distribution mimicked that of the drug-sensitive variants treated with Dox alone (Fig. 2). After treatment with Dox/NP, mean antiproliferative activity was increased in both OS cells (KHOS: Fig. 3A; KHOS_{R2}: Fig. 3B) and ovarian cancer cells (SKOV-3: Fig. 3C; SKOV-3_{TR}: Fig. 3D) in a dose-dependent manner. Dox/NP produced higher mean antiproliferative activity than Dox alone in OS cells

(KHOS: $p = 0.001$ at $0.06 \mu\text{M}$, $p = 0.044$ at $0.1 \mu\text{M}$, $p = 0.006$ at $0.3 \mu\text{M}$; KHOS_{R2}: $p = 0.001$ at $0.6 \mu\text{M}$) and ovarian cancer cells (SKOV-3: $p = 0.005$ at $0.1 \mu\text{M}$, $p = 0.005$ at $0.3 \mu\text{M}$). Dox/NP showed a five- to 10-fold higher activity at the 50% inhibitory concentration (IC₅₀) compared with the free form of Dox. Dextran-lipid nanoparticles were not cytotoxic by themselves at the dose used in this study (Fig. 3E).

The MDR cells in both cell lines showed resistance to both PLD and free Dox. Dox/NP showed an almost twofold higher mean antiproliferative activity at the IC₅₀ than PLD in both KHOS_{R2} cells (IC₅₀: $0.43\text{--}0.94 \mu\text{M}$) (Fig. 4A) and SKOV-3_{TR} cells (IC₅₀: $0.48\text{--}0.82 \mu\text{M}$) (Fig. 4B). Dox/NP showed higher mean antiproliferative activity than PLD in both KHOS_{R2} cells ($p = 0.011$ at $0.6 \mu\text{M}$, $p < 0.001$ at $1 \mu\text{M}$, $p = 0.014$ at $3 \mu\text{M}$) and SKOV-3_{TR} cells ($p = 0.023$ at $0.6 \mu\text{M}$).

In KHOS_{R2} cells incubated with AF488-tagged siMDR1/NP, fluorescence microscopy revealed bright green fluorescence in most cells within 2 hours after transfection (Fig. 5). The green fluorescence was more intense after 4 hours than at 2 hours but slightly attenuated after 24 hours. P-gp expression was confirmed in the KHOS_{R2} and SKOV-3_{TR} cell lines (Fig. 6A). After 48 hours of transfection, a 100-nM concentration of siMDR1/NP suppressed the expression of P-gp in KHOS_{R2} at levels comparable to those of the commercial transfection reagent (Fig. 6B).

Fig. 2 Images show subcellular distribution of Dox in drug-sensitive OS and ovarian cancer cells (KHOS and SKOV-3) and their MDR counterparts (KHOS_{R2} and SKOV-3_{TR}) under fluorescent microscopy.

An increase in fluorescence was observed in the MDR cancer cells treated with Dox/NP.

Fig. 3A–E The antiproliferative effects of Dox alone and Dox/NP as assessed by MTT assay are compared in drug-sensitive OS and ovarian cancer cells [(A) KHOS and (B) SKOV-3] and their MDR counterparts [(C) KHOS_{R2} and (D) SKOV-3_{TR}]. Dox/NP showed five- to 10-fold higher antiproliferative activity at the IC₅₀ in both the drug-sensitive and -resistant cell lines. Dox/NP showed higher activity than Dox alone in both OS cells (KHOS: $p = 0.001$ at 0.06 μM , $p = 0.044$ at 0.1 μM , $p = 0.006$ at 0.3 μM ; KHOS_{R2}: $p = 0.001$ at 0.6 μM) and ovarian cancer cells (SKOV-3: $p = 0.005$ at 0.1 μM , $p = 0.005$ at 0.3 μM). Values are given as mean $\pm$ SD. (E) The cytotoxicity of nanoparticles on KHOS, KHOS_{R2}, SKOV-3, and SKOV-3_{TR} was analyzed by MTT assay. The nanoparticles were not cytotoxic by themselves at the dose used in this study. The concentration of nanoparticles included in 10 μM Dox/NP was equivalent to 0.03 mg/mL nanoparticles.

Discussion

To reverse MDR, nanoparticles have the potential for encapsulating anticancer drugs and genes with the ability to passively target solid tumor tissues by exploiting the leaky vasculature and impaired lymphatic drainage system in tumors. This phenomenon is called the enhanced permeability and retention effect, which is a conventional pathway for the preferential drug accumulation in solid tumors [19, 28]. There are reports of dramatic increase in drug accumulation at the site of tumors compared with other vital organs based on this mechanism [33]. We therefore addressed three questions in this study: (1) Can Dox/NP reverse MDR? (2) Does the antiproliferative effect of Dox/NP have a better therapeutic outcome than PLD? And (3) can siMDR1/NP modulate MDR?

We acknowledge limitations to our study. First, although the clinical application of nanoparticles should be considered, our results are based on in vitro study. In vitro toxicity may not necessarily correlate with clinical response to chemotherapy in humans. In vivo studies with animal models are needed to confirm the clinical potential of our findings. Even in patients with OS, the correlation between P-gp expression and clinical outcome is still controversial [3, 18, 40, 42, 58]. Additionally, it has been reported that P-gp immunostaining does not necessarily correlate with the level of *MDR1* gene expression [20]. Second, there are obstacles in delivering siMDR1 in vivo. For instance, P-gp is also expressed in various normal tissues for cellular defense and protection, although at relatively lower levels [7, 23, 26, 44, 46, 53]. Especially in the kidney, the inhibition of P-gp can decrease the renal

Fig. 4A–B The antiproliferative effects of Dox/NP and PLD as assessed by MTT assay are compared in MDR OS [(A) KHOS_{R2}] and ovarian [(B) SKOV-3_{TR}] cancer cells. Dox/NP showed an increased antiproliferative effect compared with PLD in both MDR cells. The cytotoxicity of Dox/NP was almost two times higher than that of PLD (IC₅₀ in KHOS_{R2} and SKOV-3_{TR} with Dox/NP: 0.43 and 0.48 μM,

respectively; IC₅₀ in KHOS_{R2} and SKOV-3_{TR} with PLD: 0.94 and 0.82 μM, respectively). Dox/NP showed higher activity than PLD in both KHOS_{R2} cells ($p = 0.011$ at 0.6 μM, $p < 0.001$ at 1 μM, $p = 0.014$ at 3 μM) and SKOV-3_{TR} cells ($p = 0.023$ at 0.6 μM). Values are given as mean $\pm$ SD. Dotted line represents the 50% decrease of absorbance.

Fig. 5 Images show the effect of AF488-tagged siMDR1/NP on KHOS_{R2} cells under fluorescence microscopy after 2, 4, and 24 hours of incubation. LipofectamineTM RNAiMAX Transfection Reagent

and AF488-tagged siMDR1 alone were used as positive and negative controls, respectively. AF488-labeled siMDR1/NP were efficiently incorporated into KHOS_{R2} cells.

clearance and prolong the elimination half-life of drugs [16]. Third, the current dextran-based nanoparticles are not conjugated with active targeting ligands. Nanoparticles coupled with targeting ligands such as antibodies or

peptides that can bind to specific tumor cells can thus increase the bioavailability of drugs at the tumor site and decrease the residual toxicity [51]. Currently, epidermal growth factor receptor-targeted polymeric nanoparticles

Fig. 6A–B Images show the effects of siMDR1/NP on the P-gp expression of drug-sensitive OS and ovarian cancer cells (KHOS and SKOV-3) and their MDR counterparts (KHOS_{R2} and SKOV-3_{TR}). **(A)** P-gp expression was confirmed in the KHOS_{R2} and SKOV-3_{TR} cell lines. LipofectamineTM RNAiMAX Transfection Reagent and siMDR1 alone were used as positive and negative controls, respectively. **(B)** A 100-nM concentration of siMDR1/NP suppressed P-gp expression in KHOS_{R2} cells as effectively as siMDR1 + LipofectamineTM RNAiMAX Transfection Reagent but more effectively than the negative control siRNA or nanoparticles only.

that actively target MDR-associated phenotypes have been reported and several targeted nanoparticles have been investigated in preclinical studies for cancers [30, 50]. Furthermore, the development of combination therapies that inhibit MDR and deliver cytotoxic drugs has been recently investigated [8, 29, 38]. Liposome-based nanoparticles can be used as effective codelivery vehicles for Dox and siRNA and cause the simultaneous suppression of drug efflux pumps and antiapoptotic cellular defense combined with cell death induction [38]. Minicells were used to deliver siRNA or small hairpin RNA, followed by the cytotoxic drug. This strategy inhibited the growth of drug-resistant tumor in vitro and in vivo [27].

We first devised a strategy to develop a biocompatible nanoparticulate system to safely deliver Dox and siMDR1 to cancer cells. We chose low-molecular-weight dextran as our base polymer. Dextran is reportedly a neutral and biocompatible polymer for synthesis of nanoparticles [49]. Further, the lack of systemic toxicity is important for drug delivery applications, especially in diseases like cancer. However, dextran in itself cannot self-assemble in aqueous solution and hence it must be hydrophobically modified to facilitate self-assembly. We have previously observed that the end group functionality of the lipid tail, its carbon chain length, and the type of chemistry used to conjugate the lipid to dextran polymer can dictate the type of (hydrophilic or hydrophobic) payload/drug that could be encapsulated/loaded in such delivery systems [1, 48, 49]. In this regard, we observed short lipid chains conjugated to

linear dextran polymers using click chemical ligations were suitable for encapsulating low-molecular-weight anthracycline drugs [1]. Thus, for the purpose of stably encapsulating Dox in nanoparticles, a C₈-lipid (bromo-octane)-modified dextran derivative was synthesized using click chemistry [1]. It is proposed that the aromatic hydrophobic groups in Dox could form hydrophobic-hydrophobic interactions with the triazole ring present on click-based dextran-lipid derivatives [1]. On the other hand, we found a lipid-modified dextran synthesized by the Michael addition reaction facilitated the encapsulation of oligonucleotides and siRNAs [48]. We thus synthesized a C₆-lipid (hexyl amine)-conjugated dextran by the Michael addition reaction to enable siMDR1 encapsulation. In this case, the amine residues on the dextran-lipid chains are proposed to electrostatically interact with the negatively charged siRNA molecules. DLS and zeta potential measurements indeed proved both the functionally variant dextran derivatives encapsulating the Dox or siMDR1 resulted in formation of stable nanosized particles with slightly negative to neutral surface charge. To evaluate the intracellular distribution of Dox/NP, we conducted in vitro cell uptake measurements using fluorescence microscopy. The intracellular sites of localization of the free form of the anthracycline drug is distinctly different in several MDR cancer cells compared with their drug-sensitive counterparts [17, 22, 39, 57]. Namely, anthracycline fluorescence is reportedly found mainly in the nucleus in sensitive cells, whereas it is distributed throughout the cytoplasm of

resistant cells [22]. Interestingly, in our study, Dox distribution in MDR OS and ovarian cancer cells mimicked that of sensitive cells when MDR cells were treated with Dox/NP. These observations are consistent with previous reports and indicate dextran-based nanoparticles enabled the delivery of Dox to the nucleus even for the MDR variants [49]. Additionally, our study demonstrated a lower concentration of Dox/NP exhibited more potent antiproliferative activity against both drug-sensitive and -resistant cancer cells. Thus, it seems administration of lower doses of chemotherapeutic agents using biocompatible nanoparticle-based delivery systems could offer the clinical possibility of reducing systemic side effects, overcoming the issue of dose limitation, and reduction in the chances of development of the MDR tumor phenotype.

To date, liposomal delivery systems have been widely used as nanocarriers for drugs or siRNAs because of their simplicity, biocompatibility, biodegradability, and high loading for in vitro delivery of both hydrophilic drugs and siRNAs [37, 38]. PLD is a commercial liposomal Dox that prolongs the circulation of the drug in blood and potentiates intracellular drug accumulation. Clinically, PLD has been introduced in the salvage treatment of patients with recurrent ovarian cancer, while PLD does not induce objective remissions in patients with sarcoma [12, 36]. In our study, our formulation demonstrated better antiproliferative effect on both OS and ovarian cancer cells than PLD, indicating the dextran-based nanoparticulate delivery system may yield better clinical prospects than PLD, especially in sarcoma.

Reversal of drug resistance by siRNA targeted against the mRNA for *MDR1* has been reported for several cancer cells [11, 32]. However, there are some obstacles for delivering siRNA in the clinical setting, as described in our Introduction. We have successfully encapsulated siMDR1 in biocompatible dextran-lipid nanoparticles [48]. In KHOS_{R2} cells incubated with AF488-tagged siMDR1/NP, fluorescence microscopy revealed bright green fluorescence from the intracellularly trafficked siRNA within 2 hours of incubation and the signal could be detected even 24 hours after transfection. Our results also showed siMDR1/NP efficiently suppressed the expression of P-gp in KHOS_{R2} cells. These observations indicate that the siMDR1 was efficiently incorporated into the cells, resulting in the modulation of P-gp expression.

Our findings indicate biocompatible dextran-lipid nanoparticles could serve as a safe and promising platform, not only for Dox delivery, but also for siRNA delivery, which can effectively modulate the downregulation of P-gp in MDR cells. The development of nanoparticles for in vivo delivery is warranted and currently underway in our laboratory.

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